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Research Article

Prevalence of Selected Periodontopathic Microbes in Chronic Periodontitis Using Salivary Analysis

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Abstract

Aim: The aim of this study was to evaluate the prevalence of selected periodontopathic bacteria and HSV1 and HSV2 using a commercially available salivary analysis system.

Materials and Methods: 69 patients were consecutively recruited for the study. They were grouped into healthy (Group A), gingivitis (Group B) and chronic periodontitis (Group C) categories. Microbiological analyses were conducted for the presence of eleven periodontopathic bacteria and HSV1 and HSV2 using a salivary analysis methodology.

Results: The bacterial profile was slightly different in Group C as *P.g*, *T.f*, and *T.d* which are considered as the predominant periodontal pathogens had lower log values than other microbes. However, the log values for *P.g*, and *T.f*, were higher compared to Groups B and C. Bacterial load for *P.m* was 2.75x higher in Group C. HSV 1 was detected in one patient with chronic periodontitis whereas HSV2 was not identified in any of the participants.

Conclusion: This investigation reiterated the role of selected putative periodontopathic bacteria in chronic periodontitis with a slightly different distribution which might be explained by the geographic, genetic and ethnic variations of the study population. This study failed to demonstrate an etiologic role for HSV1 and HSV2 in chronic periodontitis.

Keywords: *Periodontitis; Bacteria; Human Herpes Simplex Virus; Saliva; India; Prevalence*

Introduction

Periodontitis is an inflammatory disease caused by a complex interaction between the host immunoinflammatory defense mechanisms and periodontopathic microbiota. Over 700+ microbial species are reported to exist in the oral cavity [1,2]. Among these 700+ microbes at least 30 separate taxa of bacteria are considered to be putative periodontal pathogens [3]. The 1996 World Workshop in Periodontics consensus report on the pathogenesis and microbial factors in periodontal diseases noted there was sufficient data to consider *Aggregatibacter actinomycetemcomitans* (*A.a.*), *Porphyromonas gingivalis* (*P.g.*) and *Tannerella forsythia* (*T.f.*) as "etiologic agents" in periodontitis [4]. Nevertheless, it is well documented that the mere presence of these bacteria is not adequate to initiate periodontal disease.

In recent years, a possible role for viruses in increased susceptibility for the development of periodontitis has been explored. Among the viruses studied, all belonging to the herpes virus family, specifically human cytomegalovirus, Epstein-Barr virus and Herpes Simplex virus (HSV1 and HSV2) have been reported to be associated with both chronic and aggressive periodontitis [5-9]. Contreras., *et al.* [10] suggest that co-infection with the Herpes virus might diminish the capability of the host cells to adequately resist bacterial challenges and thereby dictate severity of the disease.

There is strong evidence showing that the initiation of chronic periodontitis is dependent on a microbial challenge. In spite of this evidence, periodontal pathogens are often found at healthy sites [11]. This ubiquitous presence of putative periodontal pathogenic bacteria in both health and disease leads to the question of host response and ultimately genetic susceptibility. Numerous studies and systematic reviews have evaluated the role of genetics as a risk factor for both chronic and aggressive periodontitis [12-15]. It should be noted, in this regard, that not all evidence on the issue of genetic susceptibility to periodontal disease is supportive of the relationship [16]. Even with the discordant opinions and research findings regarding genetics as a risk factor for developing periodontal disease, the genetically controlled expression of inflammation-promoting cytokines, e.g. IL-1 and IL-6, in response to a microbial challenge has been hypothesized to play a prominent role in the initiation and progression of periodontitis [17]. The fact remains, however, that the initiation and progression of inflammatory periodontal disease requires the presence of bacteria or possibly, in some cases, a virus.

Thus, the purpose of this study was to conduct a cross-sectional study of the periodontopathic microbial profile, including HSV1 and HSV2 using a commercially available salivary analysis protocol, in consecutive patients presenting to the restorative and periodontal departments of a major dental institute in the country of India.

Materials and Methods

Patient population

A total of 69 consecutive patients that satisfied inclusion criteria were entered into the study. They were selected from the restorative and periodontal departments, CKS Teja Dental institute, India. The intent was explained in detail to all eligible subjects prior to the sample collection. An informed consent was obtained from all the participants. The study protocol was approved by the Ethical Committee of the NTR University of Health and Medical Sciences.

Inclusion criteria

Subjects included in the study were ≥ 18 years old and presented with ≥ 20 teeth, excluding third molars. Further, patients were included only if they had not received any subgingival periodontal therapy or periodontal surgery within the preceding 12 months. Thorough radiographic and clinical evaluation was done prior to selection for the study.

Exclusion criteria

A history of substance abuse, diabetes, rheumatoid arthritis, osteoporosis, Paget's disease, Sjogren's syndrome, any usage of systemic antibiotics, anti-inflammatory drugs, immune suppressants in the last 12 months, pregnant and lactating women, immunologic disorders including HIV/AIDS were reasons for exclusion from the study.

Documentation of periodontal parameters

Two examiners were calibrated and they reached 99% and 98.8% concordance within ±1 mm for probing depth (PD) and clinical attachment level (CAL) measurements respectively. A manual UNC periodontal probe was used for PD and CAL documentation. Gingival index (GI) and plaque index (PI) scores were obtained for all qualifying patients using Loe and Silness [18] and Silness and Loe [19], respectively. The flow diagram in figure 1 demonstrates the sequence of events required to execute the study.

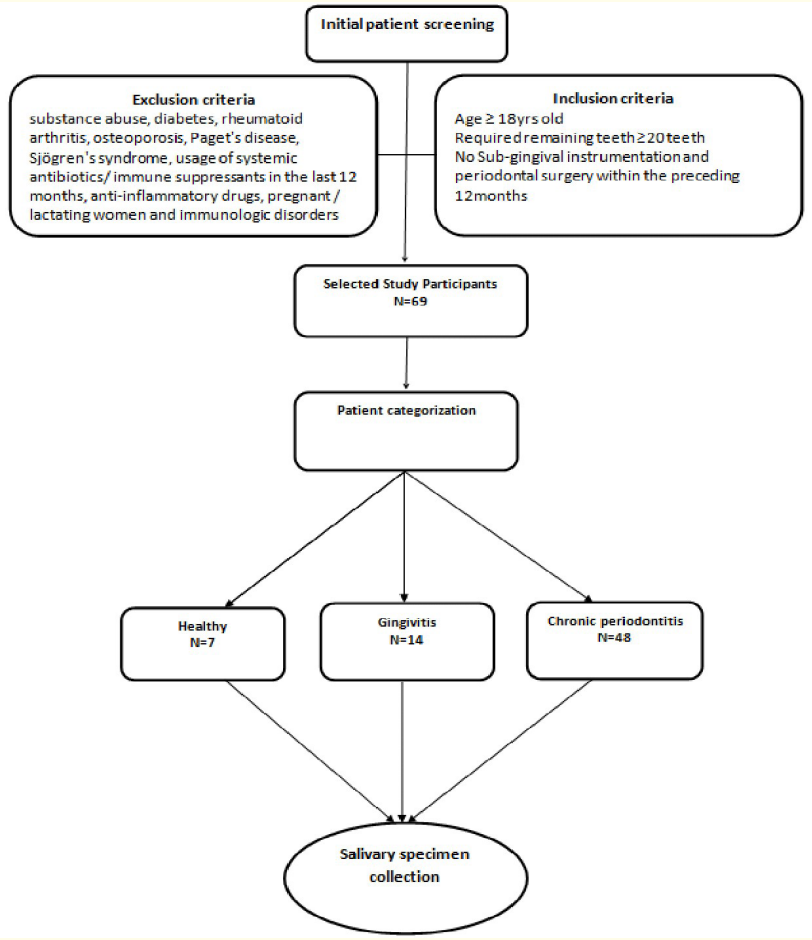

Figure 1: *Shows the sequence of events required to execute the study.*

Description of patient categories

Subjects were categorized into healthy, gingivitis and chronic periodontitis based on the 1999 International World Workshop for a Classification of Periodontal Disease and Conditions [20].

Healthy (Group A): Subjects had PD ≤ 3 mm, CAL < 2 mm, GI of < 1 and showed no evidence of radiographic alveolar bone loss.

Gingivitis (Group B): Subjects in this group had PDs and CAL similar to that of the healthy group and also did not have radiographic evidence of alveolar bone loss. However, the gingivitis group had a GI ≥ 1.

Chronic Periodontitis (Group C): Subjects assigned to this group exhibited at least 6 non-contiguous sites with PD > 6 mm, CAL ≥ 5 mm, and bone loss > 40%.

Salivary sample collection

Salivary samples were collected from all participants. None of the subjects had used an anti-microbial rinse in the 24 hours prior to collection of salivary samples. A commercially available salivary analysis kit (Oral DNA Labs, A service of Access Genetics, 7400 Flying Cloud Drive, Suite 150, Eden Prairie, Minnesota 55344-3720, U.S.A.) consisting of a sealed test tube with 5 mL of saline solution (0.9% sodium chloride), a funneled collection tube, a cap, and two bar code labels. Subjects were advised to swish with the saline solution for 30 seconds. Following swishing, the solution was expectorated into the funneled collection tube. The funnel was discarded and the collection tube was sealed with the provided cap. A bar code label with the participant's name and DOB was affixed and the collection tube was then stored in a cryogenic freezer. Samples were shipped to the commercial service laboratory for DNA-PCR analysis to determine presence of the putative periodontal pathogens and Herpes Simplex viruses.

Evaluation of putative periodontal bacteria and HSV1 and HSV2 analyses

The evaluation of periodontopathic bacteria and Herpes Simplex virus, consisting of 11 putative bacterial species and HSV1 and HSV2, was conducted by using the commercially available salivary analysis kits obtained from the same company referenced above. The method for detecting the presence of specific microbes includes genomic DNA extraction from the submitted salivary samples followed by asymmetric multiplexed polymerase chain reaction (PCR) amplification with primers and molecular beacons designed to specific gene regions of each bacterial species. Following amplification, fluorescent endpoint detection was done to provide a semi-quantitative analysis of patient sample concentration for each bacterium. Herpes Simplex virus was detected by using primers unique to HSV1 and HSV2. Final HSV results were analyzed by the peak size, height and concentration data generated through fluorescence detection by automated electrophoresis.

Statistical analysis

Data consisting of gender, age, GI, PI, mean PD, mean CAL, presence or absence of HSV1 and HSV2, and detection levels of 11 different putative periodontopathic microbes (*Aggregatibacter actinomycetemcomitans* [*A.a.*], *Porphyromonas gingivalis* [*P.g.*], *Tannerella forsythia* [*T.f.*], *Treponema denticola* [*T.d.*], *Eubacterium nodatum* [*E.n.*], *Fusobacterium nucleatum* [*F.n.*], *Prevotella intermedium* [*P.i.*], *Campylobacter rectus* [*C.r.*], *Parvimonas micra* [*P.m.*], *Eikenella corrodens* [*E.c.*] and *Capnocytophaga* species [*C.s.*]) were stored on an Excel spread sheet (Microsoft Corporation, One Microsoft Way, Redmond, WA 98052-7329, U.S.A.). Descriptive statistics regarding prevalence rates for the various microbes and Herpes Simplex viruses for each diagnostic group (health, gingivitis, chronic periodontitis) were determined. A definitive analysis for statistical significance was not attempted due to discrepancies in numbers of patients in the health and gingivitis groups compared to the number of chronic periodontitis patients and lack of an overall equitable gender distribution.

Results

The study was performed during the period of August 2013 to September of 2014. Of the 69 patients qualifying for the study, 7 were determined to have a healthy periodontal status, 14 were considered to have gingivitis and 48 subjects were diagnosed with chronic periodontitis. The study group was comprised of 51 men and 18 female participants whose ages ranged from 18 - 65 years. Study subjects reside in semi-urban and rural areas in the Southern part of India.

	Group A Mean ± SD	Group B Mean ± SD	Group C Mean ± SD
Number of Patients	7	14	48
Mean Age and Range (yrs)	22 (18 - 28)	30 (20 - 62)	48 (19 - 65)
Plaque Index and S.D.	0.66 ± 0.11	1.22 ± 0.69	2.17 ± 0.31
Gingival Index and S.D.	0.67 ± 0.13	1.44 ± 0.72	2.18 ± 0.33
Mean PD (mm)	2.25 ± 0.26	2.73 ± 0.56	3.97 ± 0.60
Mean CAL (mm)	1.25 ± 0.28	1.48 ± 0.37	4.21 ± 0.65

Table 1: *Demographic and periodontal clinical parameters of the study population.*

Demographic and periodontal clinical parameters were shown in table 1. Plaque and gingival indices were highest in Group C which were 2.17 ± 0.31 and 2.18 ± 0.33, respectively. In the chronic periodontitis category, probing depths were recorded at a total of 6238 sites and among them 1372 sites had a PD ≥ 6 mm and 22% of sites had a PD consistent with severe chronic periodontitis.

Microbial Analysis

Table 2 shows the prevalence of the eleven bacterial species in the 3 study groups using log-scale values. Collectively, the subjects in Group A (Healthy) exhibited the presence of 10 of the 11 presumed periodontopathic bacterial species. However, the Red Complex microbes, (*P.g., T.f.* and *T.d*.) had a relatively low prevalence in the healthy group compared to the gingivitis and periodontitis groups. *A.a.* was observed in one healthy subject and *P.g.* was detected in none of the healthy subjects as shown in figure 2.

Periodontal Condition	AA	PG	TF	TD	EN	FN	PI	CR	PM	EC	CS
Group A (Healthy)	0.53	0.00	4.34	3.61	2.88	2.06	2.09	3.23	6.42	4.63	5.08
Group B (Gingivitis)	1.43	1.24	4.29	4.42	4.77	2.6	3.3	5.23	6.84	4.94	4.83
Group C (Chronic Periodontitis)	2.19	3.97	5.97	4.14	6.38	5.44	5.66	6.18	19.18	4.73	6.43

Table 2: *Prevalence of microbial species (log-scale values) for healthy subjects and those with gingivitis or chronic periodontitis.*

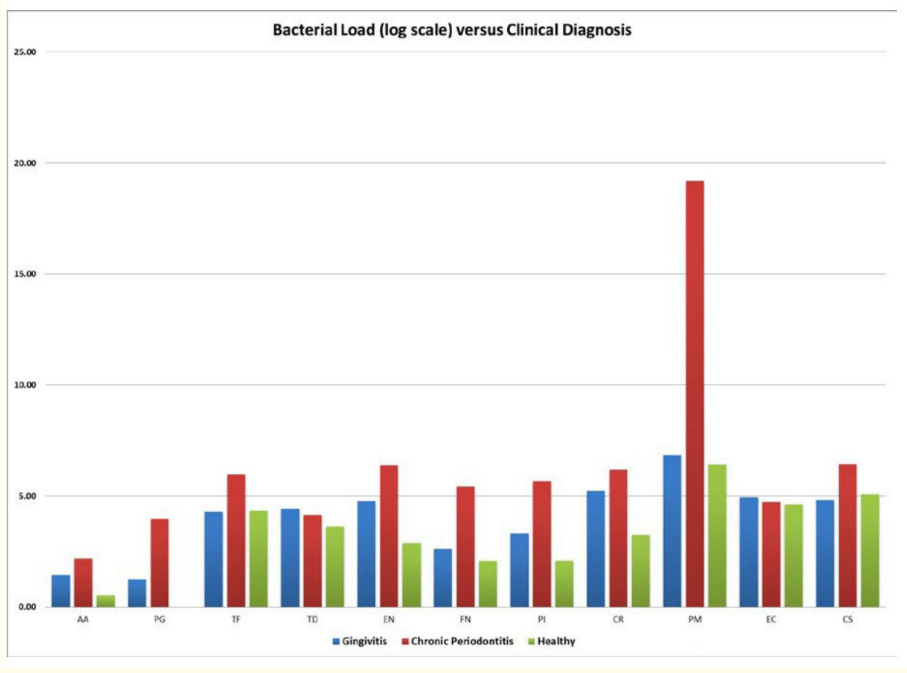

Figure 2: *Prevalence of periodontopathic bacteria in health, gingivitis and chronic periodontitis.*

The Group B (Gingivitis) subjects exhibited the presence of all 11 bacterial species putative periodontal pathogenic bacteria. This subject group harbored a greater prevalence of 9 of the 11 bacterial species, the exceptions being *T.f.* and *C.s.* which were slightly higher in the healthy group. It was interesting to note that *P.m*. showed a higher prevalence when compared to other bacterial species for both the healthy and gingivitis groups with little difference between the two groups (log scale values of 6.42 vs. 6.84 for healthy vs. gingivitis).

As might be expected, subjects in Group C (Chronic Periodontitis) exhibited all 11 bacterial species with a greater log scale numbers for 9 of the putative pathogens. The two exceptions were *T.d.* and *E.c*., both being elevated in the gingivitis group compared to the periodontitis group (log-scale values of 4.14 for *T.d.* and *4.73* for *E.c*. in chronic periodontitis vs. 4.42 and 4.94, respectively for the same microbes in gingivitis). As in both the healthy and gingivitis groups, *P.m*. was a dominate microbe in the periodontitis subjects. However, in the periodontitis subjects, *P.m.* had a prevalence of more than 2.75 times compared to the other two study groups (log scale values of 6.42 vs. 6.84 vs. 19.18 for healthy vs. gingivitis vs. periodontitis). When comparing microbial prevalence in chronic periodontitis group to both health and gingivitis, table 2 shows a ≥ 1 log difference for *P.g., T.f., E.n., F.n., P.i., P.m.* and *C.s.* of interest was the finding that the bacterial load of *P.g, T.f, and T.d* (Red Complex), considered major periodontal pathogens, was lower than other measured bacterial species within the same disease category.

Lastly, when subjects were assigned to 10 year increment age groups a tendency was noted for increased prevalence of the putative pathogenic microbes with increasing age (Figure 3).

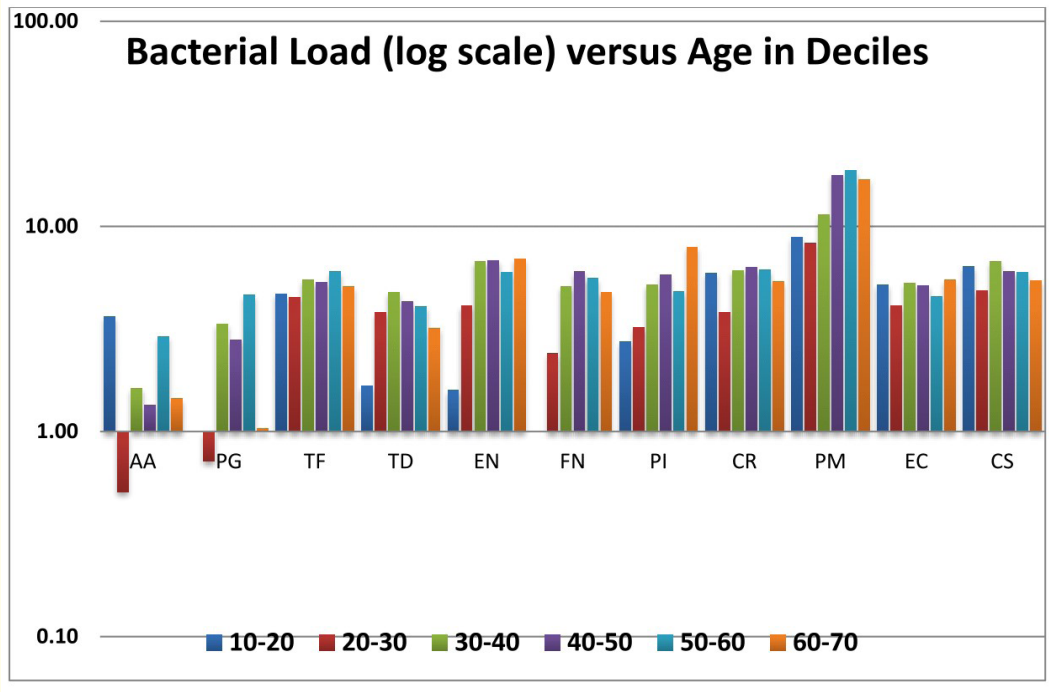

Figure 3: *Tendency for elevation of microbial levels with increasing age of study group participants.*

The results for HSV1 and HSV2 were shown in table 3. It was interesting in that HSV1 was identified in only one periodontitis subject and HSV2 was not detected in any of the subjects, regardless of their disease category.

Disease category	HSV1	HSV2
Group A (Healthy)	0	0
Group B (Gingivitis)	0	0
Group C (Chronic Periodontitis)	1	0

Table 3: *Number of patients, by disease category, in which HSV1 and HSV2 were detected.*

Discussion

This study was conducted to examine the prevalence of 11 putative periodontal pathogenic bacteria and HSV1 and HSV2 in consecutive patients presenting to a University dental clinic in Southern India. One problem with entering consecutive qualifying subjects into a study was the distribution within predetermined disease categories. The study population in this study was skewed to favor males over females (51 vs. 18, respectively) and chronic periodontitis over a diagnosis of gingivitis or health (48 vs. 14 vs. 7, respectively).

Several microbiological studies have considered *P.g*., *T.f.*, *T.d*. and *Selenomonas noxia* (*S. noxia*) as the predominant periodontopathic bacteria in chronic periodontitis [11,21]. Although *S. noxia* was not a target microbe in this study, relatively high log-scale values were recorded in the periodontitis subjects for *P.g,* and *T.f.* compared to other two groups.

P.g. has frequently been promoted as the main bacterial pathogen in chronic periodontitis [22-24]. The current study would appear to agree that *P.g.* is a prime promoter of disease in that it was detected at increasingly higher levels going from health (log-scale value = 0), to gingivitis (log-scale value = 1.24) to periodontitis (log-scale value = 3.97). Interestingly, the articles that designate *P.g*. a "keystone" periodontal pathogenic microbe also note that it's numbers need not be great in order to initiate the sequence of pathogenic phenomena associated with periodontal disease [23,24].

The observation in the current study of notably elevated levels of *P.m*. (log-scale value of 19.18 in periodontitis subjects) supports a previous study by Sanz., *et al.* [25] in which Dutch subjects showed a high prevalence of *P.m*. in the periodontitis group. A basic reason for using log-scale values to reflect prevalence of a microbe resides in the fact that bacteria multiply by fission and therefore exhibit an exponential growth phase. This begs the question as to why did the periodontitis subject group in the current study exhibit *P.m.* 2.75x greater prevalence over that of healthy and gingivitis subjects? The simplest explanation would be a regional difference in the expression of disease, i.e. populations in Southern India and The Netherlands [25] may express different ratios of species within oral microbial biofilms. A second explanation may lie in the sampling method of using saliva to predict the relative prevalence of a subgingival bacterial species in periodontitis versus a disease that is primarily characterized by supragingival biofilms, i.e. gingivitis.

Few studies report the presence of putative periodontal pathogens in healthy subjects [26]. In the current study, the healthy group subjects exhibited all the target microbes, excepting *P.g.* but with lower log-scale values than either the gingivitis or periodontitis groups. Many studies have associated *A.a.* with periodontitis, especially the aggressive form of the disease [27]. However, few studies have reported the presence of *A.a*. in a healthy population [28]. The variations in the prevalence of *A.a*. between various population groups may be due to the geographic or genetic or ethnic diversity [29]. For example, in a few ethnic groups like Asians and Hispanics, *A.a*. has been reported as a normal oral commensal with no signs of periodontal disease. A possible explanation for this latter observation and for the finding of *A.a.* in the healthy and gingivitis groups in the current study may be that the serotype of *A.a*. is not that is normally associated with periodontitis [30,31].

Even though several studies have suggested HSV as a potential pathogen in periodontitis [5,8-10,32] several reports have taken a contradictory position [33,34]. In the current study, only one patient in the periodontitis group was shown to harbor the HSV1. Again, this seeming conflict with the literature may simply be a manifestation of geographic, regional or ethnic variation. Or, the salivary analysis methodology may not be sensitive enough to measure low levels of HSV1 and/or HSV2 [35].

Conclusions

The results of this study re-emphasize the periodontopathic role of the 11 target microbes in the commercial salivary analysis technique. However, the results also failed to support a role for HSV1 and/or HSV2 in chronic periodontitis, at least in a subject population from Southern India. Future studies with a large sample size would be beneficial to better understand the prevalence of periodontopathic microbiota in this geographic and ethnic population.

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Disclosure

Dr. McGlennen is an officer in the OralDNA Lab. OralDNA Labs has provided the microbial analyses presented in this original manuscript. Other authors would like to disclose that they do not have any fianacial relationship with any entity related to the research project.

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